EXHIBIT 34

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A Supplementary Adenoviral Leader Sequence and Its Role in Messenger Translation

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Summary

During the course of lytic infection with the adenovirus 2-SV40 hybrid Ad2⁻ND1 dp2, a viral mRNA is synthesized whose 5′ end includes the start of th adenovirus 2 fiber gene and whose 3′ end lies in SV40 sequences. This hybrid mRNA codes for a protein(s) of 23-24K daltons (G. Fey, J. B. Lewis, T. Grodzicker and A. Bothwell, manuscript submitt d). Biochemical and electron microscope heteroduplex analyses reveal that two forms of this mRNA exist in the cytoplasm of cells at late tim s in infection. Both forms contain the conventi nal tripartite leader present on "late" adenovirus 2 mRNAs, but they differ in the presence or absence of a fourth leader sequence consisting of 180 nucleotides of adenovirus 2 sequences located 2,000 nucleotides upstream from the g ne. By fractionation of total poly(A)-containing Ad2⁻ND1 dp2 "late" cytoplasmic RNA in methyl mercuric hydroxide-agarose gels, we have isolated both forms of the hybrid mRNA and establish d that the presence or absence of the fourth l ader sequence has little or no influence on the ability of the messenger to direct the synthesis of the 23-24K dalton protein(s) in a cell-free translation system derived from rabbit reticulocytes.

Introduction

The genome of adenovirus 2, a double-stranded linear DNA with a molecular weight of 22.9 × 10^6 daltons (Green et al., 1967; van der Eb, van Kesteren and van Bruggen, 1969), is divided into two functional domains. One comprises the four groups of "early" genes, whose expression during the early phase of infection causes the activation of the considerably larger set of "late" genes that belong to the second domain. Most of the latter are located on the "r" strand of the viral DNA in three noncontiguous blocks which are separated from one another by intervening sets of "early" genes. These "late" genes are nevertheless transcribed from a single initiation point located about 16.3 map units from the left end of the viral DNA (Evans et al., 1977). The immediate product is believ d to be a large nuclear RNA whose 5′ end maps close to position 16.3 and whose 3′ end is copied, at least in some cases, from sequences at or near the right-hand terminus of the viral DNA.

Exactly how cytoplasmic mRNAs are forged from such precursors is unknown. As a consequence of recent work, however, we now recognize that the process involves not merely the decoration of the extremities of mRNA with poly(A) (Philipson et al., 1971) and capping structures (Moss and Koczot, 1976; Sommer et al., 1976), but also the removal of internal sequences from the nuclear precursor. This splicing procedure enables the coding sequence of each of the major late mRNAs to become coupled to a common tripartite leader derived from coordinates 16.6, 19.6 and 26.6 (Klessig, 1977; Berget, Moore and Sharp, 1977; Chow et al., 1977).

These leader sequences are found not only on authentic adenoviral mRNAs but also on mRNAs which are encoded by certain adenovirus-SV40 hybrids and consist both of adenoviral and SV40 sequences (Dunn and Hassell, 1977). The work described in this paper involved the use of several different techniques to examine the leader sequences present on a hybrid mRNA produced during infection with Ad2⁻ND1 dp2. The isolation and history of this virus and the structure of its genome have been described in detail elsewhere (E. Lukanidin and J. Sambrook, manuscript submitted). We need to trace its pedigree only as far back as Ad2⁻ND1, one of a series of nondefective hybrids between adenovirus 2 and SV40 isolated by Lewis and his colleagues. The genome of Ad2⁻ND1 contains an insertion of SV40 DNA approximately 900 nucleotides in length, in partial replacement of the segment of adenoviral DNA which maps between positions 80.6 and 86 (Crumpacker et al., 1971; Morrow and Berg, 1972; Kelly and Lewis, 1973; Henry et al., 1973; Lewis et al., 1973). Ad2⁻ND1, unlike adenovirus 2, grows equally well in simian and human cells because the integrated SV40 sequences specify a helper function (Lewis et al., 1969; Kimura, 1974; Grodzicker et al., 1974). Grodzicker, Lewis and Anderson (1976) have isolated a number of mutants of Ad2⁻ND1 which have lost the ability to grow efficiently in simian cells because they contain chain-terminator mutations in the gene coding for helper protein. From one such host-range mutant, H71, an ochre (Grodzicker, Lewis and Anderson, 1976; Gesteland et al., 1977), E. Lukanidin and J. Sambrook (manuscript submitted) selected a revertant, Ad2⁻ND1 dp2, that was able to replicate again in simian cells. The genome of Ad2⁻ND1 dp2 (Figure 1) retains the original SV40 DNA sequences that are present in H71, and has acquired a second insertion of SV40 DNA separated from the first by 350 nucleotide pairs of

adenovirus 2 DNA. At late times, the cytoplasm of cells infected with Ad2⁻ND1 dp2 contains large amounts of a polyadenylated mRNA comprised of both adenoviral sequences and SV40 sequences contributed by the right-hand insertion (see Figure 1). This RNA specifies a protein of molecular weight 23–24K daltons which carries helper activity (Tjian, Fey and Graessmann, 1978), and is composed of N terminal sequences homologous to those of adenoviral fiber protein and C terminal sequences identical to those of SV40 T antigen (G. Fey et al., manuscript submitted).

It has already been shown that adenoviral fiber mRNA has a fourth or "y" leader component located at map coordinates 78.6–79.1 in addition to the tripartite leader (Chow and Broker, 1978). Since the sequences of the 23–24K hybrid mRNA in Ad2⁺ND1 dp2 include the start of the adenoviral fiber gene, we were interested in learning whether the hybrid mRNA contains the same set of leaders. This report uses several methods to show that the population of Ad2-SV40 hybrid mRNAs generated at late times in infection with Ad2⁻ND1 dp2 consists of two species which are identical in their coding capacity and contain the common tripartite leader, but differ from one another by the presence or absence of an untranslated fourth leader sequence.

Results

Size Fractionation of Ad2⁻ND1 dp2 RNA in Methyl Mercuric Hydroxide-Agarose Gels

Preliminary characterization of late Ad2⁺ND1 dp2 RNA has been carried out by size fractionation in agarose gels supplemented with methyl mercuric hydroxide (CH_3HgOH) (Bailey and Davidson, 1976). This system causes denaturation, and single-stranded DNA and RNA can be analyzed in the same gel where each migrates according to molecular weight and independently of conformational factors. We have exploited this advantage and used restriction enzyme fragments of viral DNA as size markers to determine molecular weights for viral RNA species.

Cytoplasmic poly(A)-containing RNA (mRNA) prepared from cells at late times (23 hr) after infection with Ad2⁻ND1 dp2 was fractionated by electrophoresis through vertical slab gels cast with 1% agarose, 5 mM CH_3HgOH. The resulting gel (Figure 2) contains several bands, each of which represents a discrete size class of mRNA. The molecular weights of individual mRNA species relative to adenovirus 2/Hind III and SV40/Hae III fragments of DNA have been derived from the linear relationship that exists between electrophoretic mobility and the logarithm of molecular weight which extends over the range of fragment sizes utilized in these experiments. As we will show, with the exception of the contaminating 18 and 28S ribosomal RNAs, each band present in the ethidium bromide-stained gel contains viral sequences.

Analysis of Ad2⁻ND1 dp2 cDNA

We have used several different methods to examine the leader sequences associated with the Ad2-SV40 hybrid mRNAs generated during the course of lytic infection with Ad2⁻ND1 dp2. The first of these, sandwich hybridization (Dunn and Hassell, 1977), depends upon the fact that of the late RNAs present, only the hybrid RNAs contain both SV40 and

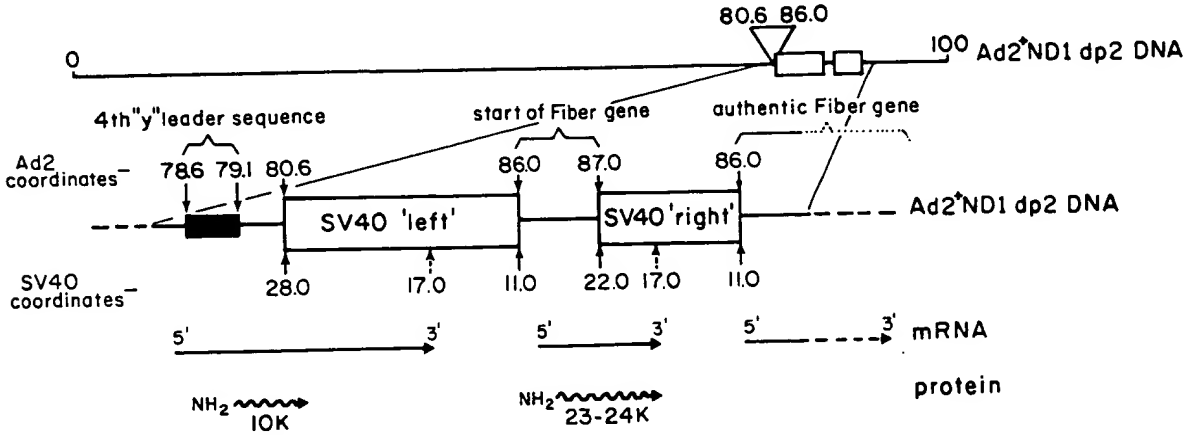


Figure 1. The Genome Structure of Ad2⁺ND1 dp2

The DNA structure is from the data of E. Lukanidin and J. Sambrook (manuscript submitted). The two insertions of SV40 sequences are shown as open boxes. Coordinates of the adenovirus 2 deletion are shown at the top of the figure. The coordinates of a fourth leader (component "y"), which forms the basis of the present study, are shown in the expanded part of the figure. The map positions of two hybrid mRNAs composed of covalently linked adenoviral and SV40 sequences are shown at the bottom of the figure, as is the protein that each hybrid mRNA specifies. The hybrid mRNA associated with the left-hand insertion of SV40 sequences in Ad2⁻ND1 dp2 specifies a 10K protein as the result of an ochre mutation in the viral genome. Details of the derivation of Ad2⁺ND1 dp2 are given in the text.

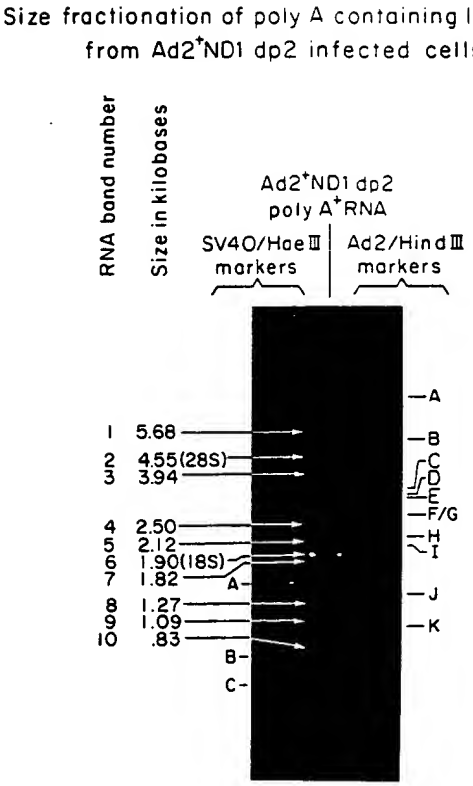


Figure 2. Analysis of Late (23 Hr) Cytoplasmic Poly(A)-Containing RNA from Cells Infected with Ad2⁺ND1 dp2 by Electrophoresis in CH_3HgOH-Agarose Gels

Cytoplasmic poly(A)-containing RNA isolated from 5 × 10^7 cells infected with Ad2⁺ND1 dp2 at an moi of 5–10 pfu per cell was loaded on a vertical slab gel cast with 1% agarose, 5 mM CH_3HgOH (see Experimental Procedures). Size markers are 1 μg each of Ad2 and SV40 DNA cleaved with Hind III and Hae III, respectively. Electrophoresis was carried out for 12 hr at a potential of 1.5 V/cm. The gel was stained by incubation for 30 min in a solution containing 0.5 M ammonium acetate and 0.5 μg/ml ethidium bromide.

adenoviral sequences. Thus when the hybrid mRNAs (or a cDNA copy of them) are hybridized to defined fragments of adenoviral DNA bound to nitrocellulose filters, they form duplexes through their adenoviral sequences, leaving their SV40 sequences as protruding tails. The process of annealing with ^{32}P-labeled SV40 DNA causes these tails to become labeled, permitting autoradiographic identification of the sequences of adenovirus 2 which are homologous to the hybrid mRNA.

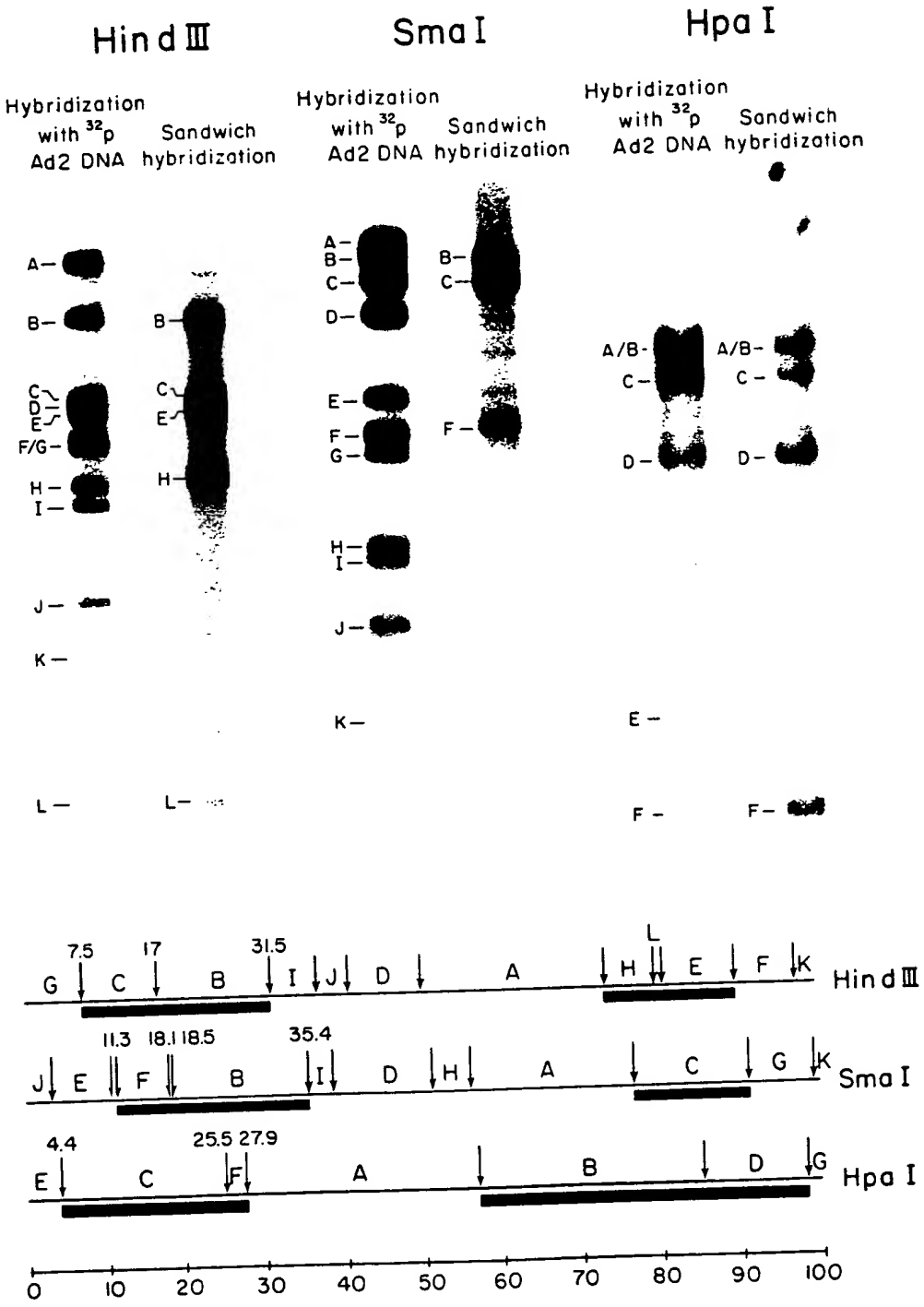
Synthesis of Ad2⁺ND1 dp2 cDNA has been carried out using total late poly(A)-containing cytoplasmic RNA as template and oligo(dT) as primer. The conditions for RNA-dependent DNA synthesis (see Experimental Procedures) are a modification of those established by other groups for similar purposes (Efstratiadis et al., 1975; Monahan et al., 1976). Preliminary experiments (data not shown), in which ^{32}P-labeled Ad2⁺ND1 dp2 cDNA was hybridized to "Southern blots" containing restriction enzyme fragments of DNA, indicated that the total cDNA was homologous to extensive regions of the viral genome which are known to code for late mRNAs.

To analyze the hybrid mRNAs specifically, we have annealed total ^{3}H-labeled Ad2⁺ND1 dp2 cDNA to Southern blots containing the Sma I, Hind III and Hpa I fragments of Ad2 DNA. The result of this experiment after sandwich hybridization with ^{32}P-labeled SV40 DNA is shown in Figure 3. It is immediately clear that only a subset of those fragments present in the original gel (left-hand strip for each enzyme) are present in the autoradiograph. For each enzyme, the bands present in the autoradiograph include the coding sequences of the Ad2⁺ND1 dp2 hybrid RNA, map coordinates 86.0–87.0 on the adenoviral genome; that is, Hind III E (80.6–89.5), Sma I C (76.8–91.0) and Hpa I D (85.0–98.5). Hybridization to DNA fragments that contain one or more of the tripartite leader sequences (Berget et al., 1977; Chow et al., 1977) is also visible in the autoradiograph. Since the restriction endonucleases have generated a series of overlapping fragments, it is possible to attribute hybridization of specific fragments to individual leader sequences contained within the cDNA; for example, Hind III C (7.5–17.0) contains only the leftmost leader (16.7), while Hpa I F (25.5–27.9) contains only the rightmost moiety of the tripartite leader (26.6).

Also visible in the autoradiograph are two additional DNA fragments which do not form part of the coding sequences of the Ad2⁺ND1 dp2 hybrid mRNA and do not contribute to the tripartite leader sequences present on many late adenoviral mRNAs. These are Hind III H (73.6–79.9) and Hind III L (79.9–80.6). This result suggests the possibility that the hybrid mRNAs, like authentic Ad2 fiber mRNA (Chow and Broker, 1978), may contain not only the common tripartite leader but also another leader sequence(s) which maps within coordinates 73.6 and 80.6 on the adenovirus 2 genome.

To examine this possibility further, unlabeled cDNA synthesized from total late poly(A)-containing Ad2⁺ND1 dp2 RNA was analyzed by electrophoresis through CH_3HgOH-agarose gels. After removal of the methyl mercury, the contents of the gel were transferred to a nitrocellulose filter (Southern, 1975) and hybridized with ^{32}P-labeled adenovirus 2 DNA (Figure 4, track 1). The ^{32}P-labeled adenovirus 2 DNA has annealed to at least eight major classes of cDNA ranging in size from 660 to 3860 nucleotides. The absence of a significant background smear in the autoradiograph indicates that the majority of the cDNA falls into discrete classes, which correspond in size to the species of viral mRNA used as templates. For each

Hybridization of ^{3}H Ad2^{+}ND1 dp2 cDNA to restriction enzyme fragments of Ad2 DNA and sandwich hybridization with ^{32}p-labeled SV40 DNA



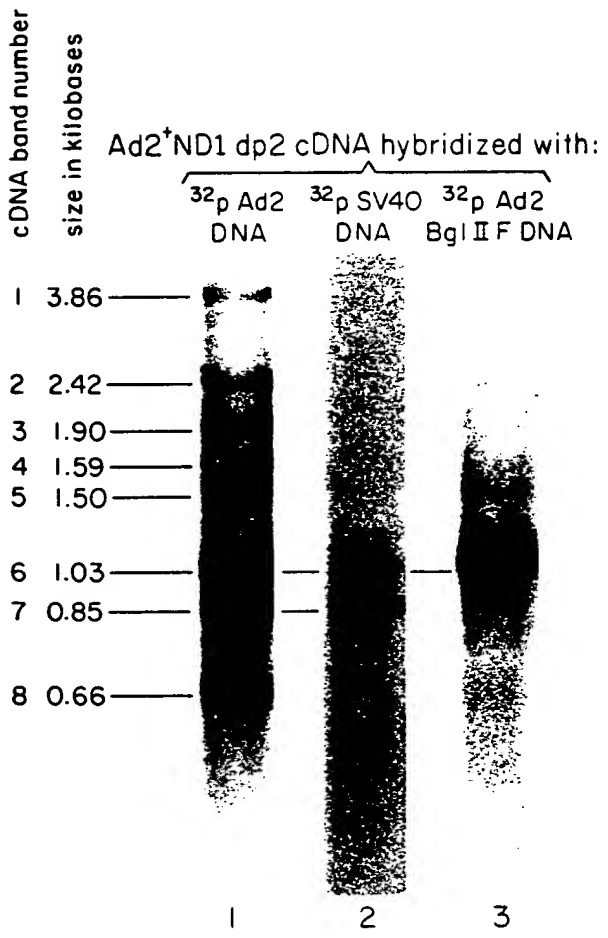


Figure 4. Hybridization of ^{32}P-Labeled DNA to Discrete Size Classes of Ad2^{+}ND1 dp2 cDNA Immobilized on Nitrocellulose Filters

0.01 μg of ^{3}H-labeled Ad2^{+} ND1 dp2 cDNA, with the template still bound, was distributed over three channels of a 1% agarose, 5 mM CH_3HgOH gel. 1 μg of adenovirus 2 DNA was cleaved with endonuclease Hind III and included in a separate track of this gel. After soaking in 0.01 M Tris-HCl (pH 7.5), 0.001 M EDTA, 0.014 M β-mercaptoethanol for 90 min at room temperature, the contents of the gel were transferred to nitrocellulose (Southern, 1975). Individual nitrocellulose strips containing immobilized cDNA were hybridized for 14 hr at 65°C with 10^7 cpm of nick-translated adenovirus 2 DNA (track 1), SV40 DNA (track 2) and Ad2 Bgl II F DNA (track 3), respectively. After washing, filters were prepared as autoradiographs using No-Screen Film type NS-54T (Kodak). Tracks 1 and 2 were exposed for 1.5 days and track 2 for 5 days. Sizes of cDNA bands were determined from the known sizes of the Hind III fragments of adenovirus type 2 DNA, which were illuminated by hybridization with ^{32}P-labeled adenovirus 2 DNA.

RNA species up to 3940 nucleotides length (bands 3–10, Figure 2), there is a corresponding cDNA band (bands 1–8, Figure 4). In each case, however, the apparent length of the cDNA is 35–320 nucleotides less than that of its presumed template, probably because the oligo(dT) molecule that serves as primer binds to the poly(A) tract at a position near the 3′ end of the coding region.

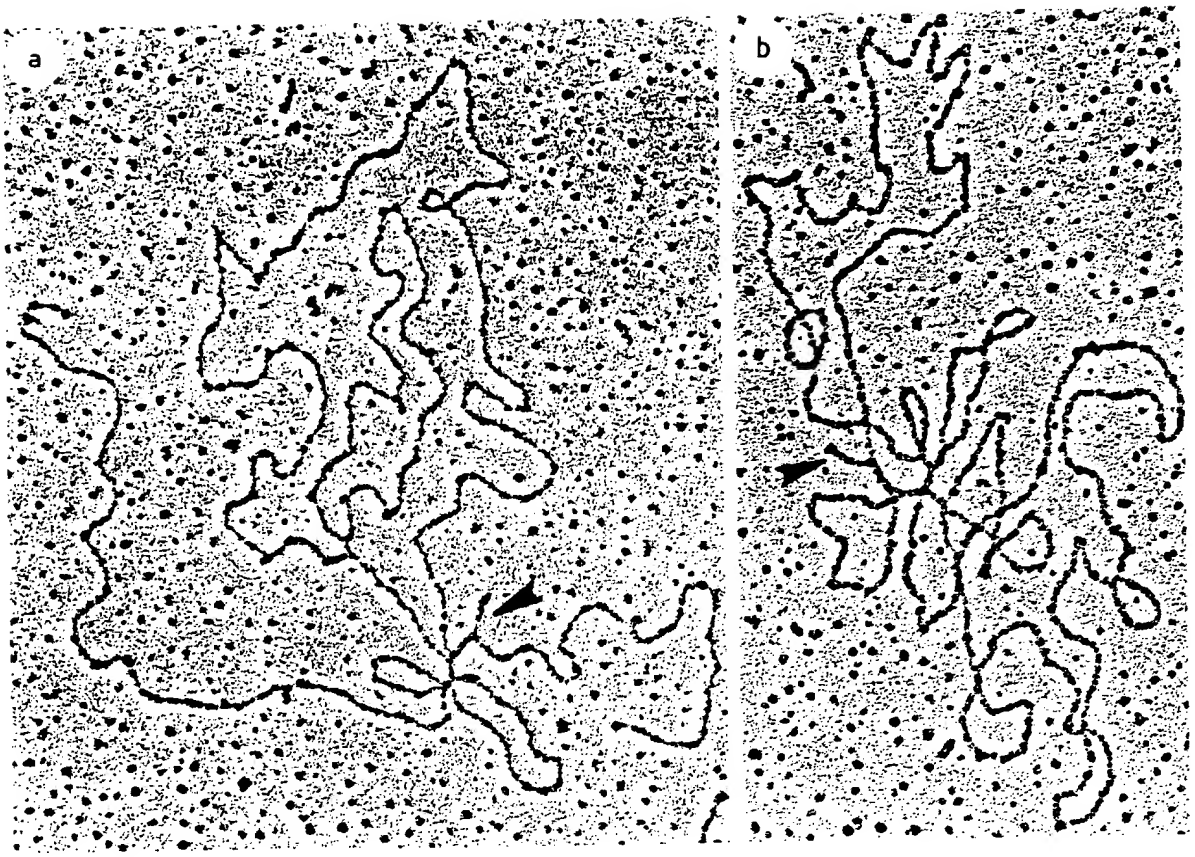
When ^{32}P-labeled SV40 DNA was hybridized to a Southern blot containing Ad2^{+}ND1 dp2 cDNA (Figure 4, track 2), we observed two prominent bands, both of which also hybridized with ^{32}P-labeled adenovirus 2 DNA. The hybrid mRNA associated with the "left" SV40 insertion in Ad2^{+}ND1 dp2 is known to be synthesized predominantly at early times in infection. In view of the approximately equal efficiency of hybridization of ^{32}P-SV40 DNA to two cDNA bands of 850 and 1030 nucleotides, and the fact that the early hybrid mRNA is known to be 1500 nucleotides in length (Flint et al., 1975; Dunn and Hassell, 1977), we considered it probable that the cDNA bands were both associated with the hybrid mRNA of the "right" SV40 insertion.

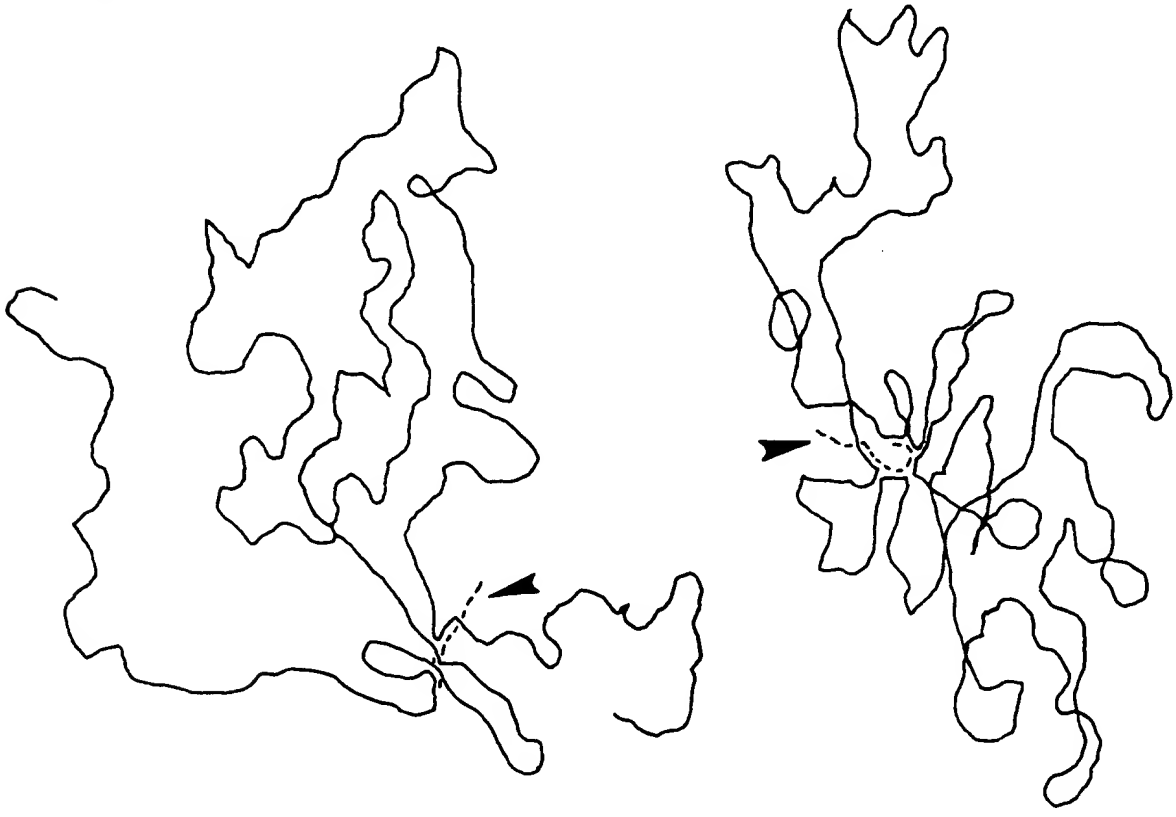
To determine the qualitative difference between the two cDNA species, we hybridized selected ^{32}P-labeled fragments of adenovirus 2 DNA to a series of nitrocellulose strips containing discrete size classes of unlabeled Ad2^{+}ND1 dp2 cDNA separated by electrophoresis through a CH_3HgOH-agarose gel. Of many fragments used, only one, Bgl II F [map coordinates 77.9–84.2 (M. Zabeau, personal communication)], showed a different pattern of hybridization to the two forms of cDNA by annealing to the larger species of cDNA (1030 nucleotides) but not to the smaller (850 nucleotides) (Figure 4, track 3). This fragment contains those adenoviral sequences which include the Hind III H/L junction to which the cDNA had already been shown to anneal after sandwich hybridization with ^{32}P–SV40 DNA (Figure 3). This region of the genome also includes the sequences which have been shown to contain the fourth leader component of adenoviral fiber mRNA (Chow and Broker, 1978).

Direct electron microscopic visualization of Ad2^{+}ND1 dp2 cDNA hybridized to the I strand (the leftward transcribed strand) of adenovirus 2 DNA revealed heteroduplexes containing either three (Figure 5a) or four (Figure 5b) single-stranded

Figure 3. Sandwich Hybridization of Ad2^{+}ND1 dp2 cDNA to Fragments of Adenovirus 2 DNA Immobilized on Filters

0.05 μg of ^{3}H-Ad2^{+}ND1 dp2 cDNA were hybridized to nitrocellulose filters containing immobilized fragments of adenovirus 2 DNA generated by cleavage with endonucleases Hind III, Sma I and Hpa I. After washing, a second round of hybridization was carried out using 1 μg of ^{32}P-labeled SV40 DNA (spec. act. 5×10^7 cpm/μg). The dried nitrocellulose filter was prepared as an autoradiograph using No-Screen Film, type NS-54T (Kodak) and exposed for 15 hr. For each enzyme, the left-hand track represents an autoradiograph of a nitrocellulose filter containing immobilized fragments of DNA after hybridization with ^{32}P-labeled adenovirus 2 DNA. Restriction enzyme maps of Ad2 DNA showing the cleavage sites of endonucleases Hind III, Hpa I (Sambrook et al., 1975) and Sma I (Mulder et al., 1974) are included in this figure. Thick lines beneath restriction enzyme fragments denote their presence in the autoradiograph.


a
b



loops, which result from hybridization of the Ad2⁺ND1 dp2 hybrid cDNA to the tripartite leader, or to the tripartite leader plus the fourth leader segment (Chow and Broker, 1978). These molecules were easily identified in the total population of cDNA-DNA hybrids by their position on the viral genome, the characteristic length of the duplex and the prominent unhybridized 5′ cDNA tail presumably composed of SV40 sequences and a poly(T) tract. Under our conditions, the I strand adenovirus 2 DNA frequently forms single-stranded circles because of the short inverted duplications at the termini of adenovirus 2 DNA (Wolfson and Dressler, 1972; Garon, Berry and Rose, 1972). It is not always possible to identify the molecular ends in such circular structures, and in these cases the map coordinates of the hybridizing segments cannot be determined. The sizes of the loops—namely 3 ± 0.3%, 7 ± 0.3% and 58.6 ± 1.3% (Figure 5a; 22 molecules measured) or 3 ± 0.3%, 7 ± 0.3%, 51.0 ± 1.5% and 7 ± 0.3% (Figure 5b; 29 molecules measured)—of the adenovirus 2 genome, and the 29.5% interval between the first and last hybridizing segments, are consistent with the expected distances between the leader segments whose map coordinates are 16.6, 19.6, 26.6 and 79.0 (Chow et al., 1977; Chow and Broker, 1978) and the gene for the 23–24K protein whose adenoviral portion maps at 86–87. Measurements of loops on the few linear heteroduplexes we observed substantiate this conclusion.

Analysis of Ad2⁺ND1 dp2 mRNA

The preceding experiments demonstrate the presence of two forms of 23–24K hybrid cDNA when poly(A)-containing mRNA is used as a template in the reverse transcription reaction. It seemed improbable that the two cDNAs arose as a result of some artifact of reverse transcription, because for each size class of cDNA there is a corresponding RNA band in the CH_3HgOH-agarose gel (Figure 2). We considered it more plausible that the two Ad2-SV40 hybrid cDNAs represent faithful copies of two templates (bands 8 and 9, Figure 2) which differ in the presence or absence of a fourth leader component. To test this hypothesis directly, we have taken advantage of a recent technological advance that allows electrophoretically separated mRNAs to be transferred from agarose gels to diazobenzyloxymethyl (DBM) paper strips (Alwine, Kemp and Stark, 1977). The sequences present in the covalently bound bands of RNA can be determined by hybridization with ^{32}P-labeled DNA probes using a method similar to that described by Southern (1975) for the analysis of immobilized DNA.

Ad2⁺ND1 dp2 poly(A)-containing late (23 hr) cytoplasmic RNA was fractionated by electrophoresis in a CH_3HgOH-agarose gel, transferred to DBM paper and hybridized with ^{32}P-labeled adenovirus 2 DNA. The major bands which appear in the autoradiograph after hybridization with ^{32}P-adenovirus 2 DNA (Figure 6, track 1) correspond to the ethidium bromide-stained bands visible in CH_3HgOH-agarose gels (Figure 2), demonstrating that the different size classes of RNA present in cells infected with Ad2⁺ND1 dp2 at late times (23 hr) after infection contain viral sequences. To identify the hybrid mRNAs consisting of adenoviral and SV40 sequences, we hybridized ^{32}P-labeled SV40 DNA to bands of RNA immobilized on paper. In the resulting autoradiograph, we observed two bands of hybridization (Figure 6, track 2, bands 8 and 9) which correspond to two species of RNA visible in stained gels (Figure 2, bands 8 and 9) and which also hybridize to adenovirus 2 DNA. To confirm that the difference between the two size classes of viral mRNA that hybridize with ^{32}P-labeled adenovirus 2 and SV40 DNAs involved the presence or absence of a fourth leader component, we again hybridized the ^{32}P-labeled Bgl II F fragment (map coordinates 77.9–84.2) to a DBM paper strip containing immobilized Ad2⁺ND1 dp2 mRNA. Only the upper band (band 8) of the two bands already shown to contain both adenoviral and SV40 sequences annealed with Bgl II F DNA (Figure 6, track 3).

The ability to isolate specific bands from CH_3HgOH-agarose gels and remove the methyl mercury (see Experimental Procedures) has enabled us to examine the RNA contained within bands 8 and 9 by forming heteroduplexes with the r strand of adenovirus 2 DNA.

The prominent heteroduplexes observed with mRNA from bands 8 and 9 contained three (Figure 7a) and four (Figure 7b) loops, respectively. The structures of these molecules correspond to those described for cDNA heteroduplexes formed with adenoviral I strand DNA. The level of cross-contamination of bands 8 and 9 was estimated to be approximately 15%, based on measurements from

Figure 5. Heteroduplexes between I Strands of Adenovirus 2 DNA and Ad2⁺ND1 dp2 cDNA

(a) Three-loop structures formed by adenovirus 2 DNA due to back-hybridization of three leader segments of the Ad2⁺ND1 dp2 hybrid cDNA at coordinates 16.6, 19.6 and 26.6 on the adenovirus genome.

(b) Four-loop structures formed by adenovirus 2 DNA due to back-hybridization of the same three leader segments plus the "y" leader of coordinate 79 on the adenovirus genome.

An illustrative tracing accompanies each electron micrograph. (——)DNA, (-----)cDNA. Arrowheads point to SV40 sequences which remain unhybridized.

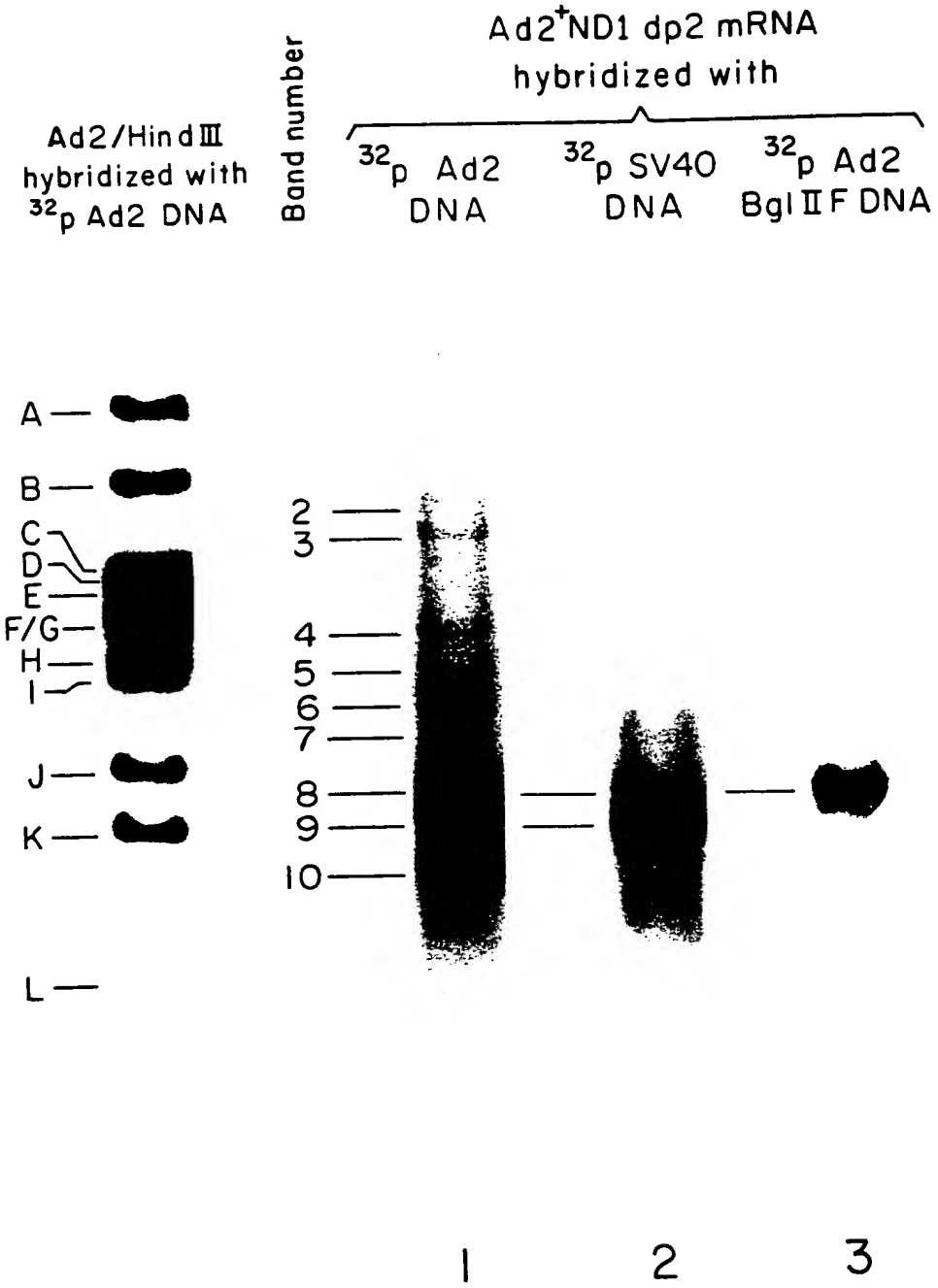


Figure 6. Hybridization of ^{32}P-Labeled DNA to Specific Size Classes of Immobilized Viral RNA

2 μg of Ad2·ND1 dp2 late (23 hr) cytoplasmic poly(A)-containing RNA were loaded onto each of three tracks of a 1% agarose, 5 mM CH_3HgOH gel. 1 μg of adenovirus 2 DNA was cleaved with endonuclease Hind III and included in a separate track of this gel. Electrophoresis was carried out for 12 hr at 1.5 V/cm, after which the methyl mercury was removed from the gel, the RNA was degraded by limited alkaline hydrolysis and the contents were transferred to DBM paper (Alwine, Kemp and Stark, 1977) as described in Experimental Procedures. Individual strips each containing immobilized RNA were hybridized for 36 hr at 42°C (see Experimental Procedures) with 5 × 10^7 cpm of nick-translated adenovirus 2 DNA (track 1), SV40 DNA (track 2) and Ad2 Bgl II F DNA (track 3), respectively. After washing, DBM strips were dried and prepared as autoradiographs using No-Screen Film type NS-54T (Kodak). Autoradiographs were exposed for 4 days.

a total of 134 and 165 molecules counted, respectively.

The only other full-length RNA species observed with any frequency was a spliced transcript complementary to the noncontiguous r strand sequences located at coordinates 4.9–6.0 and 9.6–10.9 and seen only in RNA from band 8. The possibility, first raised by Chow et al. (1977), that this RNA encodes the early 15K protein is strengthened by our observation that a polypeptide of this size is synthesized in vitro under the direction of band 8 RNA (see below).

We conclude from the biochemical data and the analysis of heteroduplexes in the electron microscope that at late times the cytoplasm of cells infected with Ad2⁺ND1 dp2 contains two forms of an Ad2-SV40 hybrid mRNA, which differ in the presence or absence of a fourth leader component.

Translational Activity of Purified Ad2⁺ND1 dp2 Hybrid mRNAs

The availability of two species of mRNA that contain identical coding sequences but differ in their complement of leader sequences allows us to investigate the role, if any, which a specific leader sequence plays in the process of translation, at least in a cell-free protein synthesizing system. The two forms of Ad2⁺ND1 dp2 hybrid mRNA (containing and lacking a fourth leader component) have been isolated from preparative CH_3HgOH-agarose gels and, after removal of methyl mercury, have been assayed in the messenger-dependent reticulocyte lysate. The result of this experiment is shown in Figure 8. The predominant product of translation of purified band 8 (track c) and band 9 (track d) mRNA migrated in SDS-polyacrylamide gels as a pair of bands with molecular weights of 23 and 24K daltons. The 23–24K protein(s) has previously been shown by G. Fey et al. (manuscript submitted) to be coded in part by the region of the Ad2⁺ND1 dp2 genome that contains the right-hand insertion of SV40 DNA, although this study did not account for the difference in electrophoretic mobility between the two forms of this protein. The purified mRNAs also direct the synthesis of minor amounts of other proteins. With the exception of a polypeptide with an apparent molecular weight of 15K, synthesized in the presence of band 8 mRNA but not seen in the products of band 9 translation (Figure 8, tracks c and d), the two purified mRNA preparations directed the synthesis of the same s t of subsidiary proteins. Similar results w re obtained using a cell-free system from wheat germ. It is significant that neither band 8 nor band 9 RNA directed the synthesis of a 10K protein previously shown by Grodzicker et al. (1976) to be th in vitro translation product of the hybrid mRNA associated with the l ft-hand insertion in Ad2⁺ND1 dp2 (see legend to Figure 1).

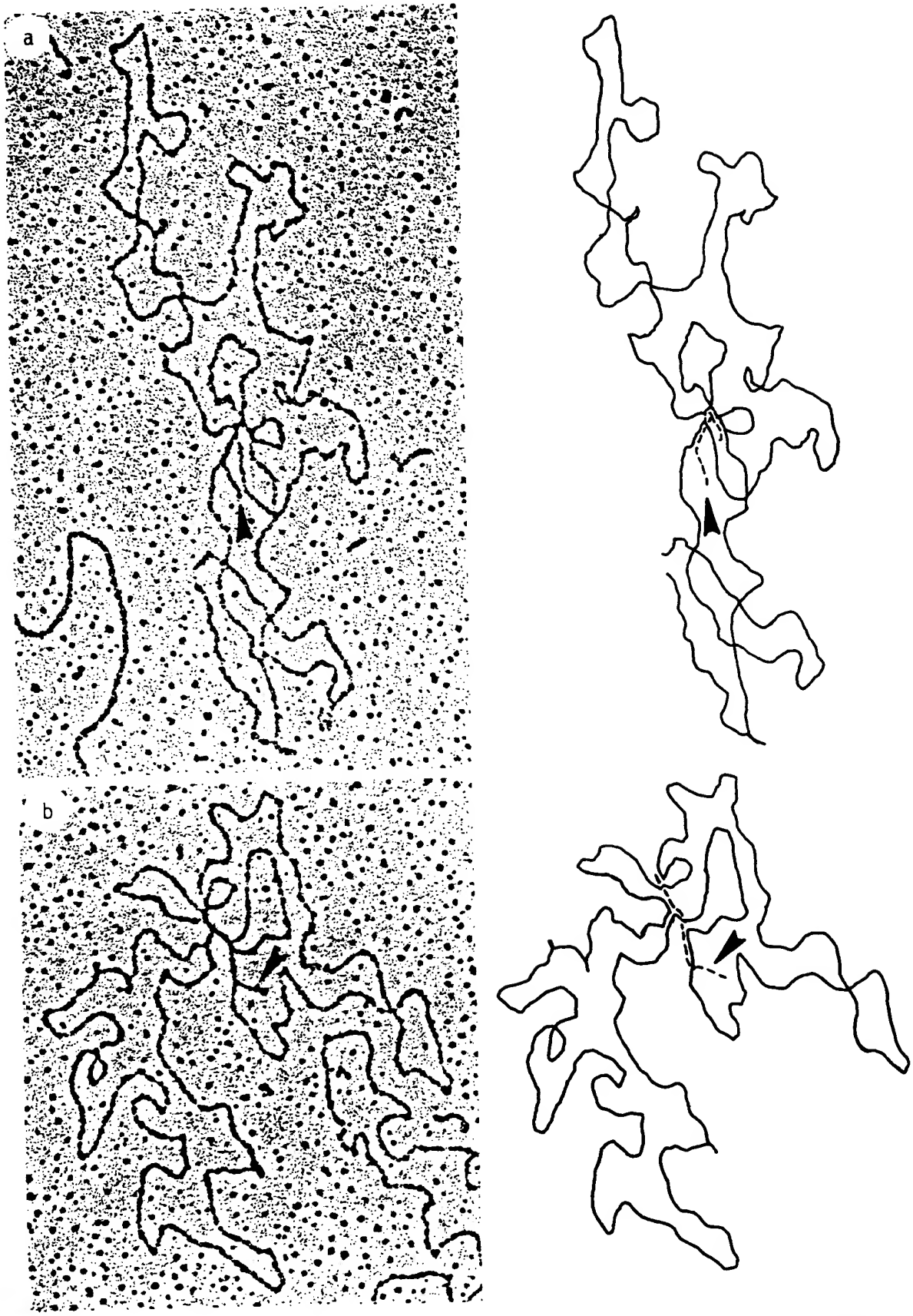
In the experiment shown in Figure 8, the RNAs from bands 8 and 9 appear to be equivalent in their ability to direct the synthesis of the 23–24K protein(s). We have, however, considered the alternative possibility that only one form of the hybrid mRNA is active in the cell-free translation system, and its presence in one band as a minor contaminant might be sufficient under conditions of mRNA saturation to create the illusion that the two forms were equivalent in translational activity. If this were the case, dilution of the mRNA would not be expected to give any reduction in the amount of product synthesized. To eliminate this possibility, we have introduced serial dilutions of both band 8 and band 9 mRNA into the reticulocyte lysate protein synthesizing system. It can be seen in Figure 8 that dilution of each mRNA leads to a corresponding reduction in the amount of 23–24K protein synthesized, suggesting that the translation reaction is not so saturated with mRNA that a minor contaminant of one band by the other could be responsible for the major 23–24K product.

From these data, we conclude that the right-hand insertion of SV40 DNA in Ad2⁺ND1 dp2 is transcribed into two species of mRNA which differ in length by 180 nucleotides and which nevertheless code for the same protein.

Discussion

We have shown by a combination of several biochemical approaches and electron microscopic heteroduplex analysis that at late times the cytoplasm of cells infected with Ad2⁺ND1 dp2 contains two distinct and separable populations of hybrid mRNAs which differ in the presence or absence of a fourth ("y") leader. By taking into account the arrangement of the Ad2⁺ND1 dp2 genome (E. Lukanidin and J. Sambrook, manuscript submitted) and the composition of the 23–24K hybrid protein (G. Fey et al., manuscript submitted), we conclude that the two forms of hybrid mRNA have the structures shown in Figure 9. At their 5′ ends are the sequences of the traditional tripartite leaders (Chow et al., 1977). Then there follow in order the optional fourth leader, the main body of the coding sequence and finally a tract of polyriboadenylic acid.

How these two closely related mRNAs are synthesized is unknown, but it seems probable that each is derived from a common precursor—the species of nuclear viral RNA which is initiated at position 16.3 on the viral r strand (Goldberg, Weber and Darnell, 1977; Weber, Jelinek and Darnell, 1977; Evans et al., 1977) and seems to extend rightwards to the end of the viral genome. There are at least two ways in which such a precursor might be treated to yield mature viral mRNAs. One way, first


a
b

detailed by Klessig (1977), requires the precursor to assume a specific configuration which brings together sequences derived from widely separated regions of the viral genome. After endonucleolytic cleavage of intervening regions, the leaders and coding sequences are covalently sealed by intramolecular ligation. In its strictest form, this model endows the leader sequences or their flanking sequences with a special function—that of directing the folding of the precursor so that splicing can occur. Alternatively, splicing of mRNA may result from a cut and patch mechanism which occurs at many points along the length of the precursor RNA. As the intervening sequences are removed, presumably in a specific order, the coding region is progressively moved closer to the 5′ end of the nascent mRNA until no sequences are left that might impede expression of the structural gene. According to this model, splicing is a multistep phenomenon with mRNAs for different structural genes being derived from the common precursor by different branching pathways. Cells at late stages of infection would consequently be predicted to contain a variety of precursor mRNA with patterns of splicing which reflect different stages and pathways of processing. In this context, Chow and Broker (1978) have identified rare forms of fiber RNA containing additional spliced leaders (leaders "x" and "z") which, like the more common "y" leader, are suggestive of intermediates in processing. It may be that the leader sequences that remain in adenoviral mRNAs have no special role in maturation of mRNA but are merely residues of that process. Perhaps they too can be entirely removed from mRNAs without loss of function. The fact that both late hybrid mRNAs appear to direct the synthesis of the 23–24K protein(s) in cell-free systems can then be viewed in two ways: either the fourth leader component has little or no effect on the translational capacity of the larger mRNA, or conversion of the larger mRNA to the smaller takes place on the polysomes. It is possible to distinguish between these alternatives by experiment.

Experimental Procedures

Cells and Viruses

Adenovirus type 2 and Ad2⁻ND1 dp2 were propagated in suspension cultures of HeLa cells (Gey, Coffman and Kubicek, 1952) in F13 medium (F13, Gibco, New York) supplemented with horse serum (5%, v/v), penicillin (100 units/ml) and streptomycin (100 units/ml).

SV40 (strain 777) was grown in CV1 cells in plastic dishes at a low multiplicity of infection (>0.01 pfu per cell) from a twice plaque-purified stock.

Isolation of mRNA

Cytoplasmic extracts were prepared from HeLa cells infected with Ad2⁻ND1 dp2 at a multiplicity of 5–10 pfu per cell. The medium was aspirated and the cells washed once in ice-cold phosphate buffered saline (PBS). After centrifugation, cytoplasmic extracts were prepared by resuspending cells in isotonic buffer [0.15 M NaCl, 0.01 M Tris-HCl (pH 7.6), 0.0015 M $MgCl_2$] and treating the suspension with 0.5% Nonidet P40 (NP40). Purification of cytoplasmic RNA was carried out as described by Craig and Raskas (1974). Selection for polyadenylated molecules was carried out by oligo(dT)-cellulose chromatography as previously described (Dunn and Hassell, 1977).

Electron Microscopy

Heteroduplex Formation between I Strand Adenovirus 2 DNA and Total Ad2⁻ND1 dp2 cDNA

The 10 μl reaction mixture containing I strand adenovirus 2 DNA (a gift from U. Pettersson) at 0.8 μg/ml, total Ad2⁻ND1 dp2 cDNA at 0.1 μg/ml, 70% formamide, 0.1 M Tris (pH 8.5) and 0.01 M EDTA (pH 8.5) was heated to 44°C for 10 min and then incubated at room temperature for 30–60 min. The mixture was transferred to ice and 3–5 μl were spread for electron microscopy using the formamide variation of the Kleinschmidt technique. For specific details of the electron microscopic procedure and data processing see Chow and Broker, 1978.

Heteroduplex Formation between Adenovirus 2 DNA and Ad2⁻ND1 dp2 mRNA Recovered from CH_3HgOH-Agarose Gels

6–8 μl of the recovered RNA was evaporated to dryness and the RNA was redissolved in 10 μl of 80% formamide, 0.1 M HEPES, 0.01 M EDTA (pH 7.9), 0.4 M NaCl containing 3.6 μg/ml native adenovirus 2 DNA. The mixture was heated to 65 ± 1°C for 10 min to denature the DNA, chilled in ice and then incubated at 40°C for 30–90 min. Reannealing was prevented by quenching with a 4 fold excess of ice cold water.

Electrophoresis in Agarose Gels Containing Methyl Mercuric Hydroxide

Gel electrophoresis of DNA markers, cDNA and mRNA was performed in vertical slab gels (Studier, 1973) of 17 × 15 × 0.4 cm, cast with 1% agarose supplemented with 5 mM CH_3HgOH (from a 1 M stock solution, 97% Alfa Chemicals, Ventron Corp., Beverly, Massachusetts) as described by Bailey and Davidson (1976). Samples were loaded in half-strength electrophoresis buffer (Bailey and Davidson, 1976) supplemented with 5 mM CH_3HgOH. At the completion of electrophoresis, DNA and RNA were visualized by ultraviolet light illumination after staining the gel for 30 min in a solution containing 0.5 M ammonium acetate (to remove methyl mercury) and 0.5 μg/ml ethidium bromide.

All manipulations involving CH_3HgOH were performed in an operating Flow hood with the operator wearing gloves.

Elution of RNA from CH_3HgOH-Agarose Gels

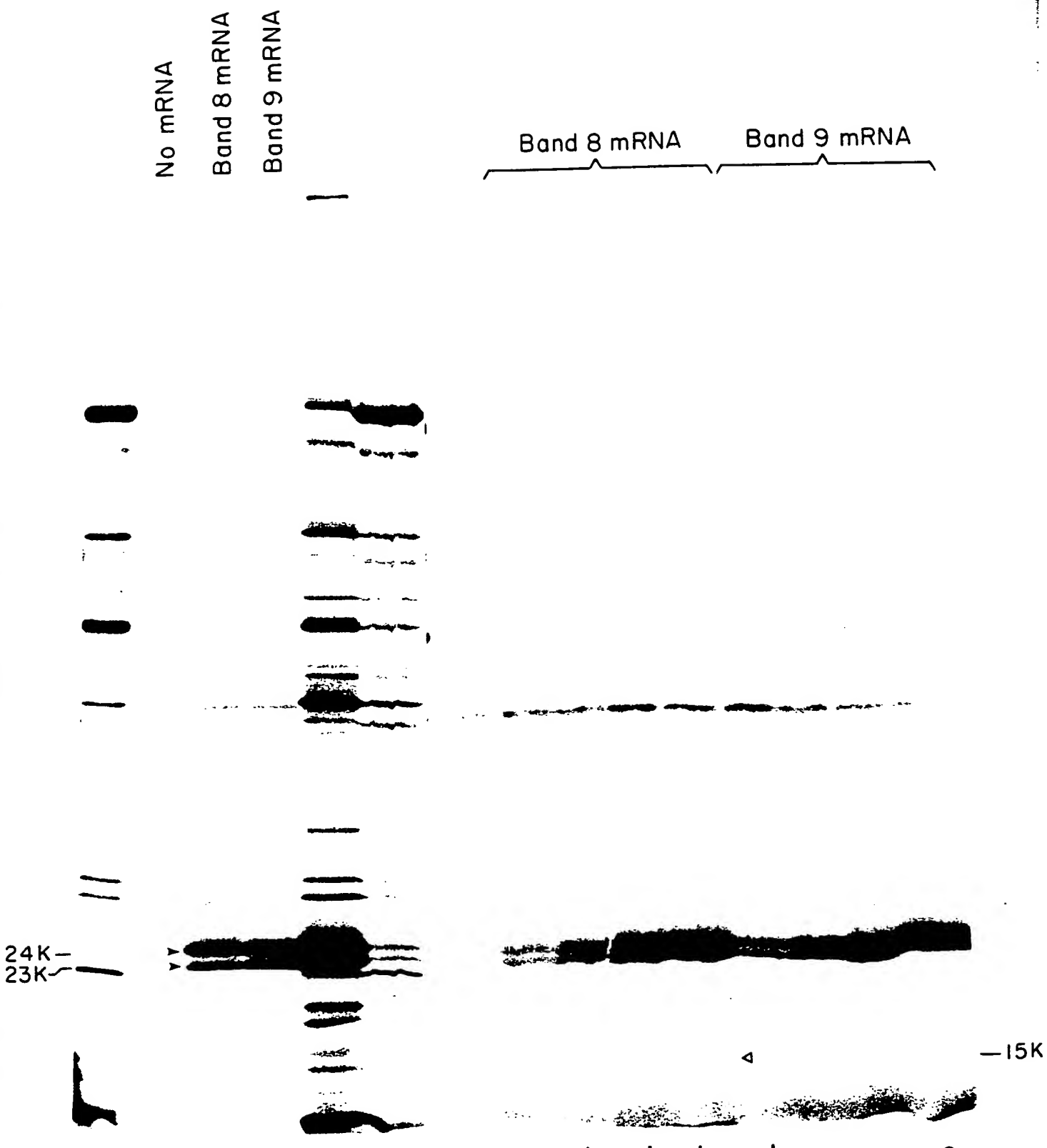
Size fractionation of RNA was carried out in CH_3HgOH agarose gels. RNA was eluted from gels in 0.15 M NaCl, 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA, 1 M β-mercaptoethanol (to remove methyl mercury) and 20 μg/ml carrier rRNA (E. coli). After three strokes in a glass homogenizer, an equal volume of phenol

Figure 7. Heteroduplexes between r Strand of Adenovirus 2 DNA and mRNA Recovered from CH_3HgOH-Agarose Gels

(a) Three-loop structures assumed by the adenovirus 2 DNA due to back-hybridization of three leader segments at 16.6, 19.6 and 26.6 with hybrid mRNA recovered from band 9 (Figure 2).

(b) Four-loop structures assumed by the adenovirus 2 DNA due to back-hybridization of the same three leader segments plus a fourth leader "y" at coordinate 79.0 with mRNA recovered from band 8 (Figure 2).

An illustrative tracing accompanies each electron micrograph. (——) DNA, (-----)mRNA. Arrows point to the SV40 sequences which remain unhybridized.


No mRNA
Band 8 mRNA
Band 9 mRNA
Band 8 mRNA
Band 9 mRNA
24K
23K
15K
a b c d e f g h i j k l m n o

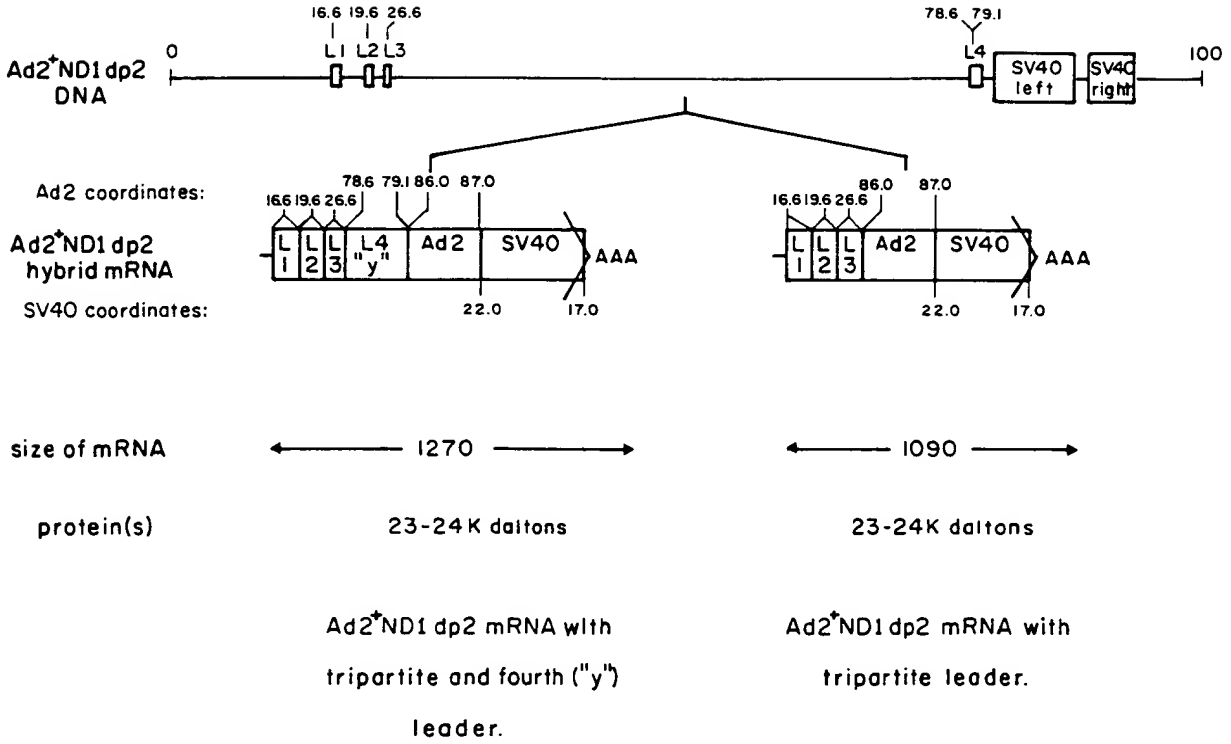


Figure 9. Structure of Two Forms of the 23–24K Hybrid mRNA

The structure of the Ad2+ND1 dp2 genome is shown at the top of the figure. A schematic representation of the two forms of the 23–24K mRNA, containing and lacking the "y" component leader, is also included. The map coordinates of the adenoviral tripartite leader sequences are taken from Chow and Broker (1977) and Berget et al. (1977), and the "y" component leader from Chow and Broker (1978).

[saturated with 0.5 M Tris (pH 7.6), 0.001 M EDTA, 0.15 M NaCl] was added and elution was allowed to proceed at 4°C for 2 hr using a rotary wheel. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected, an equal volume of fresh elution buffer was added and the process was repeated. The combined supernatants were reextracted once with phenol and once with chloroform, and the RNA was precipitated with 2 vol of ethanol at −20°C. After centrifugation, RNA was resuspended in 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA, adjusted to 0.5 M NaCl and passed over oligo(dT)-cellulose.

Cell-Free Translation

Translation of viral RNAs was carried out in a rabbit reticulocyte lysate rendered dependent upon added mRNA by incubation with micrococcal nuclease, as described by Pelham and Jackson (1976). B. Roberts provided us with this preparation. Reactions contained 10 μl of treated lysate in a final volume of 25 μl, together with 60 μg/ml rabbit liver tRNA, 20 mM HEPES-K$^+$ (pH 7.6), 80 mM K acetate, 0.85 mM Mg acetate, 2 mM DTT, 8 mM creatine phosphate, 100 μg/ml creatine kinase, 40 μg/ml hemin, 0.5 mM spermidine, 19 amino acids (methionine excepted) at 25 mM each and 1.5 mCi/ml ^{35}S-methionine (spec. act. ~500 Ci/mole). After incubation for 1 hr at 37°C and digestion with ribonuclease, 10 μl portions were subjected to electrophoresis through 7–15% polyacrylamide gels in the presence of SDS as described by Laemmli (1970). Labeled polypeptides were detected by autoradiography of the dried gels.

Reverse Transcriptase

Reverse transcriptase from avian myeloblastosis virus that had been purified by chromatography on DEAE-cellulose and carboxymethyl cellulose (Kacian and Spiegelman, 1974) was obtained from D. and J. Beard (Life Sciences Inc., St. Petersburg, Florida) through the Office of Program Resources and Logistics of the National Cancer Institute. The enzyme was purified further by sucrose gradient centrifugation (Keller and Crouch, 1972) and concentrated by three cycles of dialysis, alternating between 10 and 50% glycerol in 0.2 M potassium phosphate (pH 7.5), 2 mM dithiothreitol and 0.1% N P40 (Shell).

Synthesis of cDNA

Reaction mixtures contained in a total volume of 0.1 ml: 0.05 M Tris-HCl (pH 8.3); 8 mM $MgCl_2$; 10 mM dithiothreitol; 1 mM each of unlabeled dATP, dCTP, dGTP and TTP; 50–100 μCi of either ^{3}H-labeled dATP or dCTP (spec. act. ~40 Ci/mmole; New England Nuclear), or 50 μCi of each of the four ^{32}P-deoxynucleoside triphosphates (spec. act. ~350 Ci/mmole; Amersham-Searle); 5–50 μg of cytoplasmic poly(A)-containing mRNA; 2 μg of

Figure 8. Translation of Hybrid mRNAs in Vitro

The micrococcal nuclease-treated reticulocyte lysate was programmed with mRNA from Ad2+ND1 dp2-infected cells and the ^{35}S-methionine-labeled products were analyzed by electrophoresis in SDS-polyacrylamide gels followed by autoradiography.
Incubations contained: (b) no mRNA; (c) and (d) 10 arbitrary units of mRNA from band 8 and band 9, respectively (see Figure 2); and (e) total poly(A)-containing late Ad2+ND1 dp2 mRNA. Proteins labeled in vivo with ^{35}S-methionine late during infection with adenovirus 2 (a) and Ad2+ND1 dp2 (f) were run as markers.

The products of cell-free reactions containing progressively increasing concentrations (1, 2, 5 and 10 arbitrary units) of the purified mRNAs were resolved in a separate gel: (g) no mRNA; (h–k) band 8 mRNA; and (l–o) band 9 mRNA.

oligo(dT)$_{12-18}$ (Collaborative Research) and 25 units of reverse transcriptase. The reaction mixtures were incubated at 42°C. After 30 min, 0.2 ml of 5 mM Tris-HCl (pH 8.3), 5 mM dithiothreitol and 40 units of reverse transcriptase were added and the mixtures were incubated for an additional 30 min at 46°C. The RNA template was then hydrolyzed by incubating the mixtures for 20 min at 65°C after the addition of sodium hydroxide to a final concentration of 0.15 M. After neutralization with hydrochloric acid, the solution was passed through a column (0.5 × 25 cm) of Sephadex G-50 equilibrated with 0.01 M Tris-HCl (pH 7.5), 0.1 M sodium chloride and 1 mM EDTA. The cDNA eluted in the excluded volume fraction was concentrated by ethanol precipitation at −20°C. The precipitate was collected by centrifugation and dissolved in a small volume of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA.

Restriction Endonucleases

Endonucleases Hind III and Hae III were isolated from Haemophilus influenzae serotype d and Haemophilus aegypticus as described by Wilson and Young (1975) and Middleton, Edgell and Hutchison (1972), respectively. Endonuclease Sma I was isolated from Serratia marcescens by an unpublished method of R. Greene and C. Mulder. Endonuclease Bgl II was isolated from Bacillus globigii by an unpublished procedure of G. A. Wilson and F. E. Young. Endonuclease Hpa I was prepared according to the procedure of Sharp, Sugden and Sambrook (1973) as modified by Gallimore, Sharp and Sambrook (1974).

Blotting DNA

Agarose gels containing DNA were submerged in 0.2 M NaOH, 0.6 M NaCl for 45 min at room temperature. After rinsing in distilled water, the gels were transferred to a bath containing 1 M Tris-HCl (pH 7.4) and 0.6 M NaCl, and the DNA was transferred onto a sheet of nitrocellulose (B6, Schleicher and Schuell), essentially using the method of Southern (1975).

CH_3HgOH-agarose gels containing cDNA and DNA markers were submerged in a solution containing 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA, 0.014 M β-mercaptoethanol (to remove methyl mercury) for 30 min at room temperature. After rinsing the gel in 0.01 M Tris-HCl (pH 7.6), DNA was transferred to nitrocellulose as described above.

DNA was immobilized by baking nitrocellulose filters at 80°C for 2 hr in a vacuum oven.

Blotting RNA

CH_3HgOH-agarose gels containing mRNAs and DNA size markers were submerged in a solution containing 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA, 0.014 M β-mercaptoethanol (plus 0.5 μg/ml ethidium bromide if required) for 30 min at room temperature. Partial degradation of RNA in the gel was achieved by limited alkaline hydrolysis; that is, the gel was rocked gently for 40 min at room temperature in a solution containing 0.05 M NaOH, 0.014 M β-mercaptoethanol. The gel was washed twice (10 min each) in 200 ml of 0.2 M potassium phosphate and twice (5 min each) with 200 ml 0.025 M potassium phosphate. Diazobenzyloxymethyl paper was prepared using N-(3-nitrobenzyloxymethyl) pyridinium (BDH Chemicals) as described by Alwine, Kemp and Stark (1977). Transfer of DNA and RNA to activated paper was carried out using 25 mM potassium phosphate essentially as described by Alwine, Kemp and Stark (1977).

Radioactive Labeling of Viral DNA

Ad2 and SV40 DNA were labeled in vitro with α-^{32}P-deoxyribonucleoside triphosphates by the nick translation reaction of E. coli polymerase (Rigby et al., 1977).

Hybridization

Sandwich Hybridization

Sandwich hybridization was performed essentially as described by Dunn and Hassell (1977). Before hybridization, nitrocellulose filters were incubated for 6 hr at 65°C in a solution containing 6 × SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin (Denhardt, 1966). After removal of the template and ethanol precipitation, the pellet of cDNA was taken up in a small volume of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA. Hybridization was carried out in a final volume of 3 ml containing 6 × SSC, 0.5% SDS, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin. Nitrocellulose filters were rolled into cylinders and inserted into 150 × 25 mm test tubes. Hybridization fluid was added, the tubes were sealed with Teflon stoppers and tape and set in a horizontal position on a rotating wheel submerged in a water bath at 65°C. Following overnight hybridization (12–16 hr), the filters were exhaustively washed in 2 × SSC, 0.5% SDS for 6 hr at 65°C. At the end of this period, the washing solution was replaced with 3 ml of hybridization fluid containing 1 μg denatured ^{32}P-labeled SV40 DNA (spec. act. 5 × 10^7–1 × 10^8 cpm/μg), 6 × SSC, 0.5% SDS, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin. Hybridization was carried out at 65°C for 12–16 hr, after which filters were exhaustively washed in 2 × SSC, 0.5% SDS for 6 hr at 65°C. After rinsing in 2 × SSC, filters were air-dried, mounted on Whatman 3MM paper and prepared as autoradiographs.

Hybridization to Blots Containing Immobilized RNA

Hybridization to paper strips containing covalently bound RNA was performed essentially as described by Alwine, Kemp and Stark (1977).

Before hybridization, paper strips containing RNA were incubated for 4 hr a 42°C in a solution containing 50% formamide, 5 × SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 0.2% SDS, 1% glycine and 1 mg/ml denatured sheared calf thymus DNA. Hybridization was carried out in tubes in 3 ml of hybridization fluid containing 50% formamide, 5 × SSC, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 1 mg/ml denatured sheared calf thymus DNA and 1 μg/ml denatured ^{32}P-labeled viral DNA. Hybridization was carried out at 42°C for 36–48 hr, after which strips were exhaustively washed in 50% formamide, 5 × SSC, 0.2% SDS for 4 hr at 42°C. After rinsing in 50% formamide and 5 × SSC, strips were air dried, mounted on Whatman 3M paper and prepared as autoradiographs using No-Screen Film, type NS-54T (Kodak).

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Note Added in Proof

–We have carried out experiments to determine whether the fourth leader sequence of the hybrid mRNA is removed during translation in both the reticulocyte and wheat germ protein synthesizing systems. In each case, purified ^{32}P-labeled fourth leader-containing RNA was incubated under conditions which yielded efficient synthesis of the 23–24K protein(s). The RNA recovered from these reactions and assayed in methyl mercuric hydroxide-agarose gels was found to be unaltered in electrophoretic mobility. This result eliminates the possibility that the fourth leader sequence is spliced out in the cell-free system and strongly suggests that the fourth leader-containing RNA is translationally active in its own right.

–Recent observations in our laboratory indicate that the use of DNA size markers to calibrate methyl mercuric hydroxide-agarose gels, under the conditions reported in the present study, is satisfactory for determining the sizes of RNAs containing more than 1300 nucleotides, but may lead to overestimates with smaller RNAs.